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Mutations of the APC tumor suppressor gene are linked to a number of hyperplastic events in humans and in mice, including breast cancer. Recent evidence indicates that the APC gene product may play an active role in the **wnt-1** signaling pathway as a regulator of cytoplasmic B-catenin, a known mediator of transcription. Cytoplasmic B-catenin signaling may contribute to the transformation of cancerous cells, mediating the acquisition of a more aggressive, invasive phenotype. This project proposes to identify genes which may be regulated by B-catenin by using a novel method called gene-trapping, as well as through investigation of several "best guess" genes, including Leukemia Inhibitory Factor (LIF). Characterization of these genes will potentially elucidate another portion of the **wnt-1** signaling pathway, and give fresh insight into the exact nature of the cell cycle perturbations caused by B-catenin and other components of the pathway.

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FOREWORD

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INTRODUCTION

Cell to cell adhesion is a phenomenon often affected in cancer. Important for everything from development to cellular communication, the mechanisms of adhesion may offer clues about the nature of metastasis, invasion, and cancer progression. One important class of cell adhesion molecules is that of the cadherins. These are a family of calcium-dependent transmembrane proteins that mediate cell-cell interactions through homotypic extracellular associations.[1] They are particularly important during cellular differentiation and morphogenesis. Anchoring cadherins to the actin cytoskeleton are a second class of proteins known as catenins, three of which have been identified: α-catenin, similar to the actin-binding protein vinculin; β-catenin, homologous to the Drosophila segment polarity gene Armadillo; and γ-catenin, or plakoglobin, found in adherens and desmosomal junctions.

β-catenin itself is a 92-kD protein that contains several conserved regions known as armadillo repeats.[2] Although originally identified as a link between E-cadherin and the actin-bound α-catenin, recent studies have established β-catenin's role as not only a cell adhesion molecule but also as a signaling molecule in the *wnt-1* pathway, with putative roles in both colon and breast cancer. [3,4]

The *wnt-1* pathway, which is thought to be involved both normal development and cancer, is still under investigation. It is believed that the *wnt-1* signal indirectly leads to the down-regulation of GSK3β, a serine-threonine kinase. Normally, the absence of the *wnt* signal allows GSK3β to phosphorylate the APC gene-product, which in turn reduces cytoplasmic levels of β-catenin protein. It is thought that APC and GSK3β function in concert to control cytoplasmic β-catenin levels by targeting the protein for

degradation. In the presence of the *wnt* signal, cytoplasmic β-catenin levels remain high. Many breast and colon cancer cell lines, due to both known and putative mutations in many of the molecules of this pathway, also exhibit high levels of cytoplasmic β–catenin. In addition, recent studies have found that β-catenin interacts with the TCF/LEF family of known transcription factors.[5] This, in addition to evidence that β-catenin accumulates in the nucleus when cytoplasmic levels are increased, have lead to speculation that β-catenin serves to regulate the expression of other gene products which may be important factors in the etiology of cancer at the cellular level.[6]

The goal of this study is to identify putative downstream targets of β-catenin, and to study how these gene products predispose cells to cancerous phenotypes. In order to do this, we have used a two pronged approach: first, the application of the gene-trap technique; and second, the identification and investigation of several "best-guess" targets, based upon previous studies. One of these, Leukemia Inhibitory Factor (LIF), has yielded some encouraging results, and is being investigated further.

LIF is a multifunctional cytokine that plays a role in such diverse functions as hematopoiesis, neuronal differentiation, as well as the maintenance of embryonic stem cells in a dedifferentiated state.[7] This 20 kD secreted glycoprotein, a member of the IL-6 family of cytokines, also appears to stimulate cell proliferation in a variety of cancer types, including breast and prostate.[8] In breast cancer specifically, recent studies have shown LIF to be regulated by a number of factors, including progestins.[9] Evaluation of the promoter sequence reveals several putative TCF/LEF binding sites. This information led us to speculate that LIF might be regulated by β-catenin. Initial investigation has yielded some promising results.

MATERIALS AND METHODS

Cell Culture All cell lines used in these studies (MCF7, SW480, SKBR3) were obtained from the ATCC (American Tissue Culture Core; Rockville, MD) and were maintained at 37°C, 5% CO_2 in DMEM containing 5% Fetal Bovine Serum.

RT-PCR cDNA was synthesized using MMLV-RT and reverse primers. Subsequent PCR was performed in a PE thermocycler.

Transient Transfections Transient transfection of mammalian cells was by the calcium phosphate method. Briefly, 100,000 cells were plated, and 24 hours later fresh media was added. After 4 hours, DNA was complexed to the calcium phosphate solution and added to cells for 6 hours, followed by two washes with dPBS, and final addition of fresh media. Cells were harvested for transfection 36-72 hours after transfection, and analyzed. Cotransfection with the Renilla plasmid (Promega) allowed for control of transfection efficiency.

Promoter Luciferase Constructs LIF promoter-luciferase constructs hLIF666, hLIF274, and hLIF82 were kindly provided by Dr. AM Bamberger.[9]

RESULTS

Gene Trap The original gene trap vectors provided to us by LM Forrester[10] contained the *lacZ* reporter gene. Upon further reflection, we decided to reengineer the construct to express Green Fluorescent Protein instead of *lacZ*, as GFP expression and identification

can occur in live cells. I am in the process of this manipulation, and once the new gene-trap vector has been constructed, experiments as outlined in my original proposal will be undertaken (estimated date: October 1998).

LIF In order to establish some baseline information about which breast cancer cell lines secreted LIF, and which were responsive to it (assumed by expression of the receptor), RT-PCR was performed on 16 cell lines (HBL100, MCF7, ZR75B, T47D, MDA-MB468, SKBR3-RA, CAMA1, MDA-MB134, MDA-MB435, $MCF7_{ADR}$, MDA-MB231, BT549, A1N4, $A1N4_{myc}$, MCF10A, and MDA-MB436. The results showed that the following 8 cell lines expressed mRNA for LIF: ZR75B, MDA-MB468, $MCF7_{ADR}$, MDA-MB231, A1N4, $A1N4_{myc}$, MCF10A, and MDA-MB436. Strikingly, all 16 cell lines expressed the receptor mRNA, although at varying levels. This suggests that LIF may act in an autocrine or paracrine fashion, and that regulation of its activity is more likely through regulation of LIF expression than of the receptor.

Next, the hLIF666 promoter-luciferase construct was transiently transfected into 3 cell lines with normal (i.e. low) levels of cytoplasmic β-catenin: MCF7's, T47D's, and HS578T's. The low activity of hLIF666 when transfected with a control vector was strikingly contrasted with an approximately 7-10-fold increase in activity when β-catenin was cotransfected. These results have been repeated in triplicate >3 times.

Finally, hLIF274 and hLIF82 were used to attempt to narrow down which region of the promoter contained the actual binding site for TCF/LEF/β-catenin. Preliminary experiments point to the site being contained in the 82 bp just upstream from the start

site, but pending repetition of these experiments these results cannot be considered conclusive.

CONCLUSIONS

These preliminary studies have resulted in evidence that β-catenin may indeed regulate the expression and secretion of LIF. Before this statement can be made definitively, however, further experiments are required. First, it is important to pinpoint the exact location of the TCF/LEF/β-catenin binding site on the LIF promoter. Next, it will be interesting to find out whether APC, Calpain Inhibitor, or a dominant negative form of the TCF/LEF transcription factor have any impact on LIF promoter activity. It will also be crucial to investigate whether or not LIF protein secretion is actually increased in the presence of β-catenin. Finally, functional studies examining the role LIF may play in the breast cancer phenotype will be necessary to bring some value to this work.

These investigations, as well as the ongoing attempts to use the gene-trap approach, constitute the work being undertaken as allowed by this grant.

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[1] Pierceall, WE, AS Woodard, JS Morrow, D Rimm, and ER Fearon. 1995. Frequent alterations in E-cadherin and α- and β-catenin expression in human breast cancer cell lines. *Oncogene.* 1:1319-1326.
[2] Funayama, N, F Fagotto, P McCrea, and BM Gumbiner. 1995. Embryonic axis induction by the armadillo repeat domain of β-catenin: evidence for intracellular signalling. *J. Cell. Bio.* 128:959-968.
[3] Rubinfeld, B, I Albert, E Porfiri, C Fiol, S Munemitsu, and P Polakis. 1996. Binding of GSK3β to the APC--β-catenin complex and regulation of complex assembly. *Science.* 272:1023-1026.
[4] Gumbiner, BM. 1995. Signal transduction by β-catenin. *Curr. Opin. Cell. Biol.* 7:634-640.
[5] van de Wetering, M, R Cavallo, D Dooijes, M wan Beest, J va Es, J Loureiro, A Ypma, D hursh, T jones, A Bejsovec, M Peifer, M Mortin, and H Clevers. 1997. Armadillo coactivates transcription driven by the product of the drosophila segment polarity gene *dTCF. Cell.* 88:789-799.

[6] I Simcha, M. Shtutman, D. Salomon, J. Zhurinsky, E. Sadot, B. Geiger, and A Ben-Ze'ev. 1998. Differential nuclear translocation and transactivation potential of β-catenin and plakoglobin. *J. Cell Bio.* 141:1433-1448.

[7] Takeda, T, H Kurachi, T yamamoto, H Homma, K Adachi, K Morishige, A Miyake, and Y Murata. 1997. Alternative signaling mechanism of leukemia inhibitory factor responsiveness in a differentiating embryonal carcinoma cell. *Endocrin.* 138:2689-2696.

[8] P Kellokumpu-Lehtinen, M Talpaz, D Harris, Q Van, R Kurzrock, and Z Estrov. 1996. LIF stimulates breast, kidney, and prostate cancer cell proliferation by paracrine and autocrine pathways. *Int. J. Cancer.* 66:515-519.

[9] AM Bamberger, I Thuneke, and HM Schulte. 1998. Differential regulation of the human LIF promoter in T47D and MDA-MB 231 breast cancer cells. *Breast Canc. Res.* 47:153-161.

[10] Forrester, LM, A Nagy, M Sam, A Watt, L Stevenson, A Bernstein, AL Joyner, and W Wurst. 1996. An induction gene trap screen in embryonic stem cells. *Proc. Natl. Acad. Sci.* 93:1677-1682.